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UNIVERSITÀ DEGLI STUDI DI TORINO

Food Additives and Contaminants. Part A. Chemistry, Analysis, Control, Exposure and Risk Assessment
29:1893-1900; 2012
DOI: 10.1080/19440049.2012.719645

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Prednisolone is a synthetic glucocorticoid widely employed in bovine clinical practice, which may be also illegally used as a growth promoter. Recent in vitro and in vivo studies lend support to the hypothesis that prednisolone could be synthesized from cortisol in untreated cattle subjected to stressful events. To verify such a hypothesis, a field survey was conducted on urine samples collected from 131 guaranteed untreated cows and analyzed for the presence of prednisolone and prednisone -in some instances also for cortisol and cortisone- with a validated HPLC/MS-MS method. None of the examined samples exhibited either prednisolone levels higher than the CCα limit (around 0.70 $\mu g\ l^{-1}$) or prednisone, being therefore officially compliant for both analytes. Trace amounts of prednisolone, approximately estimated in the range 0.1-0.3 $\mu g\ l^{-1}$, were found only in 7 samples from cows also showing urinary cortisol and cortisone levels higher than those detected in negative specimens, as the result of a probable stress condition.

KEYWORDS: Prednisolone; cows; urine; cortisol, illicit growth promoters; liquid chromatography-tandem mass spectrometry

Introduction

Corticosteroids, including mineralocorticoids and glucocorticoids (GCs), represent an important class of hormones synthesized in the adrenal cortex. Among GCs, corticosterone and cortisol are reported to regulate a number of physiological processes, including stress response, inflammation, immune function, hydro-electrolyte balance, reproduction, and behaviour (Greco and Stabenfeldt 2002). Several molecules with increased pharmacological activity have been so far synthesized by introducing small modifications in the chemical backbone of physiological GCs, and are widely used in the clinical practice. For instance, the introduction of a double bond between the carbon atoms 1 and 2 of cortisone and cortisol yields prednisone and prednisolone, respectively (Figure 1). Such modifications result in both an extended duration of GC therapeutical effects and a several-fold increase in their pharmacological potency, particularly in the anti-inflammatory action. These features, combined with the absence of a parallel increase of sodium-retaining effects, enhance the suitability of synthetic GCs for therapeutic purposes (Ferguson and Hoenig 1995). A number of commercial preparations containing different prednisolone esters are currently available for administration to cattle, covering a wide range of therapeutical applications, including primary ketosis, disorders of tendons and the musculoskeletal system, allergic reactions, skin diseases, and shock. In addition, prednisolone is included in a number of antibiotic preparations for intramammary administration, indicated for the treatment of cow mastitis (McDonald et al. 2007).

Besides legal treatments, prednisolone and other GCs are frequently employed just before the animals are sold, to mask more or less severe pathologies, especially in the case of old cows at the end of their productive cycle. Another common law infringement is the administration of intramammary infusions without declaring nor applying an appropriate withdrawal time. In the last decade, synthetic GCs, including prednisolone, have been increasingly employed as illicit growth promoters in cattle (Stephany 2001; Cannizzo et al. 2011; European Commission 2012). When administered at low dosages for an extended period of time, GCs are known to significantly

improve the feed conversion ratio, to increase the area of *longissimus dorsi,* one of the most valuable muscles in bovine meat breeds, and to enhance the overall carcass quality traits, yielding pale and tender meat (Carraro et al 2009; Girolami et al. 2010). The illegal use of synthetic GCs with a strong pharmacological activity may result in the accumulation of potentially dangerous residues in meat and offal. Therefore, in the EU it is mandatory to restrict the administration of such drugs to the therapeutic indications and keep an official record of the treatment by a licensed veterinarian, who should also apply an appropriate withdrawal time to comply with the established maximum residue limits (MRLs) for edible tissues and milk (European Commission 1996).

According to Council Directive 96/23/EC (European Commission 1996), Member States must draft and implement each year a National Residue Plan to monitor the misuse and/or abuse of veterinary drugs in food producing species and animal productions. In recent years, a notable increase in bovine non-compliances for corticosteroids has been observed. Interestingly, the last published report of non-compliant results (referring to 2010), ranks Italy largely first, with a total of 38 cases, the majority of which referred to dexamethasone (30), and only 4 to prednisolone, 4 to its metabolite/precursor prednisone, and 1 to betamethasone (European Commission 2012).

As mentioned before, there is a close structural resemblance between cortisol and prednisolone on the one hand, and cortisone and prednisone on the other one. As the physiological synthesis of cortisol is reported to increase under stressing conditions (Palme et al. 2000; Greco and Stabenfeldt 2002), some researchers have recently hypothesized that the excess glucocorticoid might be partially converted to prednisolone, although the possible endogenous biotransformation pathway could not be defined (Pompa et al. 2011). The biochemical formation of prednisolone was demonstrated in artificially stressed cows by Pompa et al. (2011), and in cattle urine samples contaminated by fecal bacteria by Arioli et al. (2010). The finding of some non compliances for prednisolone in urine samples collected from officially untreated veal calves (Ferranti et al. 2011) apparently corroborated the conclusions drawn in the studies mentioned above. Taking into account

that the misuse and abuse of prednisolone in cattle is well established, it becomes important also from a forensic viewpoint to make a clear distinction between the conditions that may favour the alleged endogenous production of prednisolone and cases when the presence of prednisolone in urine samples is expected to reflect an exogenous administration. In order to verify if and to what extent prednisolone could be generated by physiological biochemical processes, a large scale field survey was carried out involving untreated cows reared in Piedmont (northern Italy).

Materials and Methods

Chemicals, reagents, and standard solutions

Prednisone, prednisolone, and cortisol were purchased from Sigma Aldrich (Milan, Italy). Triamcinolone acetonide-d_6 (Internal Standard) was obtained from RIVM (Bilthoven, The Netherlands).

Acetonitrile and diethylether were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide and hydrochloric acid were supplied by Carlo Erba Reagenti (Milan, Italy). Ultrapure water was obtained by a Milli-Q Millipore system (Bedford, MA, U.S.A.). β-glucuronidase-arylsulfatase obtained from *Helix pomatia* was purchased from Roche Diagnostics GmbH (Penzberg, Germany).

Stock standard solutions of prednisone, prednisolone, cortisone and cortisol were prepared in acetonitrile at a concentration of 1000 mg l^{-1} and stored at −20°C in the dark. Working acetonitrile solutions containing all the analytes at different concentrations were prepared by mixing the stock solutions at the proper dilution. The working solutions were used to spike negative urine samples at various levels.

Analytical method

The basic analytical method used for the determination of prednisone and prednisolone was described in a previous study (Cannizzo et al. 2011). This method had been slightly modified, in order to include the simultaneous determination of cortisone and cortisol, by adding three SRM transitions for each new analyte within the MS/MS programming, at the expected retention time windows. The rest of the analytical procedure was unmodified with respect to the published method, including sample preparation, chromatographic parameters, SRM transitions and retention time windows for the determination of prednisone and prednisolone.

In short, the sample preparation comprised the IS (Triamcinolone Acetonide D6) ~~addiction~~ addition (0.1 mg l^{-1}) to 5 ml of sample, followed by enzymatic deconjugation by means of β-glucuronidase-arylsulfatase and liquid/liquid extraction with diethylether at pH = 8.5-9.5.

The instrumental analysis was performed by an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a Merck LiChroCART – C18 (5 μm) 150 mm × 4.6 mm column and a Phenomenex SecurityGuard 4.0 mm × 2.0 mm precolumn. The chromatographic run was carried out by a binary mobile phase of water and acetonitrile, using the following programme: isocratic with 28% acetonitrile for 8 min; linear gradient from 28% to 35% in 2 min; isocratic with 35% acetonitrile for 8 min; linear gradient from 35% to 50% in 4 min; isocratic with 50% acetonitrile for 7 min. The total run time was 29 min, the flow-rate was 0.4 ml min^{-1} and the injection volume was 10 μl. The LC was interfaced to an Applied Biosystems API 4000 triple–quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in atmospheric pressure chemical ionization (APCI) – positive ion mode. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the transitions from the protonated molecular ion of each analyte to the fragment ions listed in Table 1.

Animals and sampling procedures

The study was carried out on 131 clinically healthy cows in the age range of 2.5–8 years, reared in 6 beef cow-calf enterprises and 6 dairy farms, respectively, and located in the Turin and Cuneo provinces of Piedmont (northern Italy) (Table 2). Farms were selected according to both personal (i.e. a long history of compliances not only for glucocorticoids) and official records concerning drug treatments, in addition to a breeder formal declaration that the cows had not been subjected to any drug treatment in the last 30 days prior to sampling procedures, at least. Typically, 12 cows for each farm were enrolled in the study; to take into account possible influences of the reproductive status, sampling was conducted on the following categories (n=3) : a) early pregnancy (4 months); b) late pregnancy (8 months); c) estral phase; d) anoestrus. Sampling was performed by licensed veterinarians in the morning under conditions of natural micturition, as recommended by the National Residues Plan, and preceded by a thorough cleaning of the external genitalia (labia and the ventral commissure) by means of disposable paper towels. After collection, urine samples were immediately refrigerated, frozen at -20 °C within 4 hours, and conferred to the analytical laboratory.

Statistical analysis

Where appropriate, data were compared using the Mann–Whitney's non-parametric test (GraphPad Prism® 4.0 for Windows, GraphPad Software Inc., San Diego, CA, USA) at a significance level of $P < 0.05$.

Results and discussion

Analytical performances

The present method is adopted for prednisone and prednisolone confirmation analysis by the Regional (Regione Piemonte) Veterinary Control Services in the frame of the National Residue Plan, and it is fully validated in agreement with the Commission Decision 2002/657/EC (European Commission 2002). The validation protocol is detailed elsewhere (Cannizzo et al. 2011) with CCα

values for prednisone and prednisolone of 0.66 μg l^{-1} and 0.67 μg l^{-1}, respectively. Although a careful quantification range, yielding adequate accuracy, could not be expanded below 0.5 μg l^{-1}, the limit of detection (LOD) values were approximately 0.05 μg l^{-1}, i.e. one order-of-magnitude below the CCα values. Several sensitivity tests were executed on blank urine samples spiked with the analytes at 0.5 μg l^{-1} concentration, providing an average S/N ratio exceeding 35 for both analytes. Prednisone and prednisolone concentrations in the range 0.05–0.5 μg l^{-1} were occasionally detected in the real samples, as is reported in the subsequent section. For these samples, the reported concentrations have to be considered as reasonable estimations, since their accuracy was variable beyond acceptable limits. A typical chromatogram, obtained from a blank urine sample spiked with the analytes at low concentration, is reported in Figure 2 and shows the main SRM profiles for the four analytes.

Similar figures-of-merits were obtained for cortisol and cortisone. Validation experiments were conducted on real urine samples, spiked at various concentrations, within the range 0.5 ~~e~~ and 10 μg l^{-1}. Calculated LOD values were around 0.1 μg l^{-1}. The presence of endogenous production of this analyte prevented us from verifying LOD values experimentally in real samples.

Occurrence of prednisone or prednisolone in cow urines

The aim of the present study was to ascertain whether prednisone and/or prednisolone could be detected in the urines of untreated cows, reared for milk or veal production, under regular housing conditions. In order to avoid false positive results, all the farms selected for the study were managed by breeders personally known by Veterinary Officers as scrupulously complying with the legislation in force concerning the use of drugs in food producing species (DLgs 193/2006). Other steroids (for example 17β-19-nortestosterone) have been reported to occur naturally in pregnant cows (Scarth et al. 2009). Therefore, the state of pregnancy or the estrous cycle status for each enrolled cow was assessed by means of ovary transrectal palpation performed by a licensed

veterinarian. Urine sampling was strictly performed as recommended by the National Residue Plan and particular care was taken to avoid fecal contamination, in order to exclude any possible role played by fecal bacteria in the generation of both analytes (Arioli et al. 2010).

The results of the analytical determinations evidenced that in none of the 131 examined urine samples either prednisolone or prednisone were present at a level higher than either the decision limit (CCα) value (around 0.70 μg l^{-1}) or even their limit of quantification (0.5 μg l^{-1}). Previous studies concerning the endogenic presence of these glucocorticoids in cattle urine report conflicting results. Our findings are consistent with those from a previous investigation conducted on a smaller number of Charolais and Friesian bulls (n=20), where no traces of prednisolone or prednisone could be detected in several urine samples obtained from untreated (n=14) and dexamethasone-treated (n=6) animals (Cannizzo et al. 2011). On the other hand, Tölgyesi et al. (2010) found prednisolone in the trace range of 0.3-0.9 μg l^{-1} in four bovine urine samples originating from the Hungarian residue control programme, but no specific details were provided about the origin of such samples. Finally, prednisolone at 0.5 μg l^{-1} concentration was also detected in one out of seven urine samples from experimental Friesian veal calves before starting the administration of the same glucocorticoid (Ferranti et al. 2011) .

Relationship between alleged stress and the recovery of trace amounts of prednisolone in cow urines

In the present study, urinary prednisolone was occasionally detected at levels largely below the CCα limit in a small number of non pregnant cows of either breed (n=7), with concentrations approximately estimated between 0.1 and 0.3 μg l^{-1} for three animals (Figure 3), and less than 0.1 μg l^{-1} for the remaining four individuals. Prednisone was never detected (Table 3). Interestingly, all cows with detectable urinary prednisolone were reared in loose housing systems, irrespective of the type of breeding (i.e. milk vs. beef production). The impact of housing conditions and management

procedures are known to exert variable effects on the hypothalamic–pituitary–adrenal (HPA) function in livestock. In particular, the stress experienced by the animal during urine sampling may interfere with the effect of housing or management procedure (Von Borell 2001). It has been suggested that, in beef bull breeding systems, restricting space allowances may result in chronic stress inducing slightly higher plasma cortisol levels with respect to loose housed cattle (Gupta et al. 2007; Odore et al. 2011). On the other hand, it is conceivable that the restraint procedures aimed at urine sampling may elicit an acute stress response of higher intensity in animals accustomed to free housing with respect to those reared in tether systems. A close linear correlation has been reported between unbound plasma cortisol and urinary cortisol (Lindholm and Schultz-Möller 1973) and the latter has been used to monitor HPA activity in several farm species (Mormède et al. 2007)

In order to verify whether an endogenous production of prednisolone could be attributed to alleged conditions of higher stress, total urinary cortisol and cortisone levels (free plus conjugated hormones) were determined in both urine samples showing trace amounts of the glucocorticoid (n=7) and prednisolone-negative specimens collected from cows kept in tie-stall barns (n=8). Data depicted in Figure ~~3~~ 4 demonstrate that the urines collected from cows reared in loose housing farms and containing prednisolone at a low level (< CCα) had median cortisol concentrations more than threefold higher (2.66 vs. 0.75 μg l^{-1}) than those sampled from tethered cows ($P<0.001$), whereas figures for cortisone were 1.27 vs. 0.24 μg l^{-1}, respectively ($P<0.02$, data not shown). Such findings are qualitatively consistent with the conclusions of Pompa et al. (2011). They reported that an artificial stress elicited in three cows by the i.m. administration of a synthetic analogue of the adrenocorticotropic hormone caused the urinary cortisol level to abruptly rise up to 163-238 μg l^{-1}coincident with the appearance of remarkable prednisolone concentrations (1.01-3.51 μg l^{-1}) as soon as two hours after the treatment. Similar results were observed also in urines from experimental untreated veal calves, showing that the stress induced by slaughtering procedures may entail a fourfold increase in cortisol concentration, accompanied by the detection of measurable

prednisolone levels (up to 1.4 μg l^{-1}) in 5 out of 9 individuals (Ferranti et al. 2011). From a quantitative point of view, however, a significant difference exists between our study and the previous ones, as the concentrations of prednisolone that we occasionally detected in urines never exceeded the CCα level, i.e. no non-compliant samples were yielded by the alleged endogenous production of prednisolone.

Previous studies conducted on cattle demonstrated that the observed increase of plasma cortisol in response to stressful events (e.g. truck transportation, dehorning) is matched by a parallel rise of circulating progesterone (Cooper et al. 1995; Buckham Sporer et al. 2008; Odore et al. 2011). This was attributed to a transient overwhelming of adrenal enzymes (e.g. 11β-hydroxylase) involved in the conversion of progesterone to cortisol (Chretien and Seidah, 1981). Consequently, also in the alleged endogenous production of prednisolone and possibly prednisone the adrenal origin can not be ruled out. In this respect, it should be noted that the biochemical pathways leading to the oxidation of cortisol/cortisone to prednisolone/prednisone in mammalian tissues have never been unravelled, as opposed to the biotransformations operated by certain bacteria capable of expressing a Δ1-dehydrogenase enzyme, which introduces a double bond between the carbon atoms 1 and 2 of cortisol and cortisone, respectively (Kristeva and Grigorova 1987).

In conclusion, none of the 131 cow urines collected at farm and analyzed with a method validated according to the Commission Decision 2002/657/EC (European Commission 2002) was found to contain concentrations of prednisone and prednisolone above the CCα limit - and thus officially non compliant - irrespective of the cow housing conditions and type of breeding. Trace amounts of supposedly endogenous prednisolone (approximately between 0.1 and 0.3 μg l^{-1}) together with relatively high cortisol concentrations were found in 7 urine samples from free housing cows allegedly stressed by the urine sampling operations. Further research to confirm the relationship between different stressful conditions, cortisol secretion, and prednisolone appearance in cattle urine is under way. A promising development of the present research could be the use of

the prednisolone/cortisol urinary concentration ratio as a potential mean to distinguish between exogenous administration and alleged putative endogenous production.

Acknowledgements

The project was financially supported by "Regione Piemonte–Direzione Sanità-Prevenzione Veterinaria" "Determinazione di prednisolone e suoi metaboliti in bovini da latte e da carne; biotrasformazione endogene in condizioni sperimentali di campo e di laboratorio".

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Table 1. Mass spectrometric acquisition parameters for selected reaction monitoring operating mode.

Analyte	Precursor ion m/z	Declustering Potential (V)	Product ions (Q= quantifier transition)	Collision Energy (V)	Collision Cell Exit Potential (V)
Prednisone	359.1	70	359.1 → 313.2 **Q**	19	9
			359.1 → 295.2	20	9
			359.1 → 267.2	21	7
Prednisolone	361.3	55	361.3 → 265.2	24	9
			361.3 → 279.2 **Q**	18	9
			361.3 → 223.2	29	9
Cortisone	361.2	83	361.2 → 163.4 **Q**	36	10
			361.2 → 121.3	43	10
			361.2 → 105.3	64	10
Cortisol	363.2	69	363.2 → 121.3 **Q**	33	10
			363.2 → 147.3	44	10
			363.2 → 309.4	25	10
Triamcinolone Acetonide D_6	441.4	65	441.4 → 421.3 **Q**	15	14
			441.4 → 403.4	21	13

Table 2. Breed, breeding typology and housing conditions of the cows enrolled in the study

Farm no.	Breed - Breeding Typology	Total heads	Housing	n. of sampled cows
1	Friesian–dairy enterprise	125	Tie-stall	12
2	Friesian–dairy enterprise	60	Tie-stall	12
3	Piemontese-beef cow-calf enterprise	220	Tie-stall	12
4	Piemontese-beef cow-calf enterprise	120	Tie-stall	12
5	Friesian–dairy enterprise	251	Littered loose-house	12
6	Piemontese-beef cow-calf enterprise	160	Littered loose-house	11
7	Piemontese-beef cow-calf enterprise	70	Littered loose-house	12
8	Friesian–dairy enterprise	137	Littered loose-house	12
9	Piemontese-beef cow-calf enterprise	101	Tie-stall	1
10	Friesian–dairy enterprise	185	Littered loose-house	12
11	Friesian–dairy enterprise	109	Littered loose-house	12
12	Piemontese-beef cow-calf enterprise	125	Littered loose-house Organic farm	11

Table 3. Breed, breeding typology, reproductive status, and housing conditions of the cows showing trace amounts of prednisolone in the urines

Farm no.	N. of cows showing traces of prednisolone	Reproductive status	Detected concentrations ($\mu g\ l^{-1}$)[1]	Breed and housing
5	2	1) anestrus	~ 0.2-0.3	Friesian dairy cows-littered loose-house
		2) estrus	~ 0.2-0.3	
8	1	estrus	~ 0.1-0.2	Friesian dairy cows-littered loose-house
10	1	estrus	< 0.1	Friesian dairy cows-littered loose-house
12	3	1) anestrus	< 0.1	Piemontese Beef cow calf enterpr. littered loose-house
		2) anestrus	< 0.1	
		3) anestrus	< 0.1	

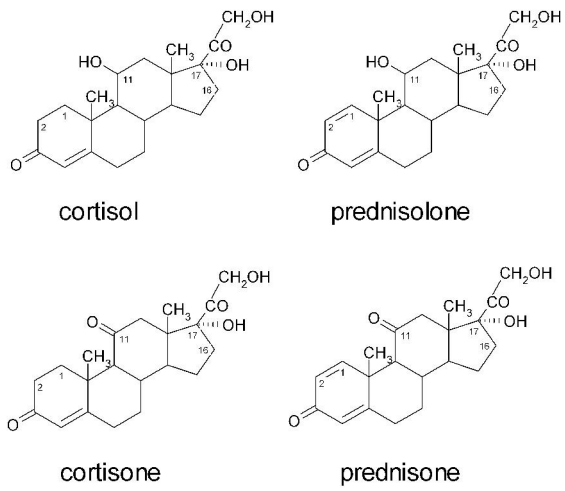

Figure 1. Structural resemblance of cortisol and prednisolone, and cortisone and prednisone.

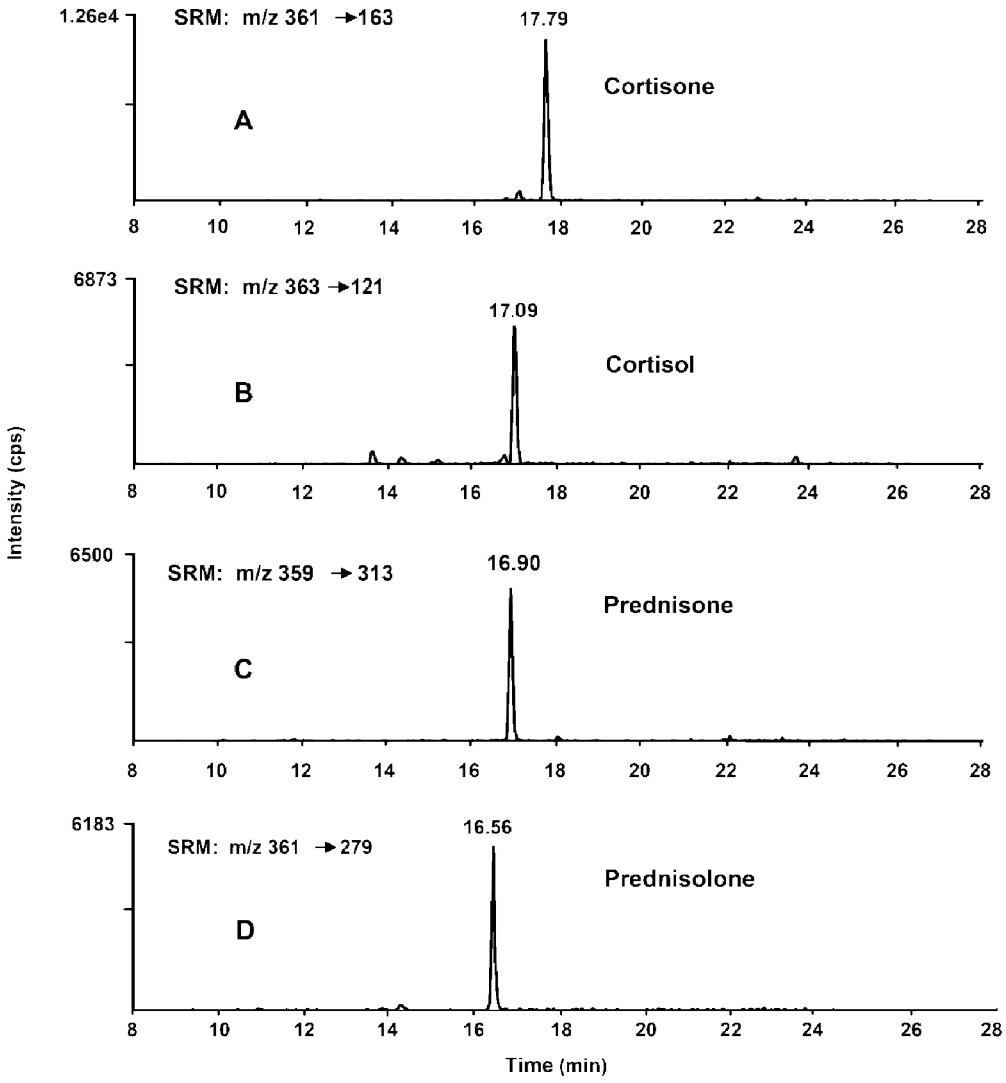

Figure 2. SRM chromatographic profiles for (A) cortisone, (B) cortisol, (C) prednisone, and (D) prednisolone, spiked at 1 ng ml^{-1} concentration in blank bovine urine. For all analytes, the quantifier SRM transition is shown.

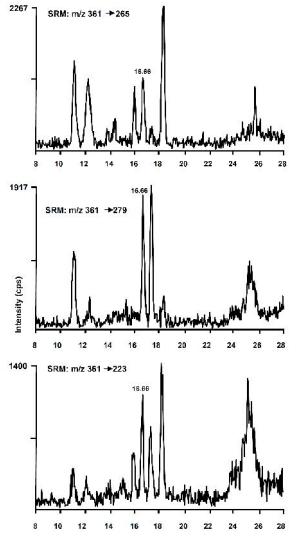

Figure 3. SRM chromatographic profiles of a urine sample showing prednisolone traces (rt 16.66). Prednisolone concentration was estimated between 0.2 and 0.3 µg l^{-1}.

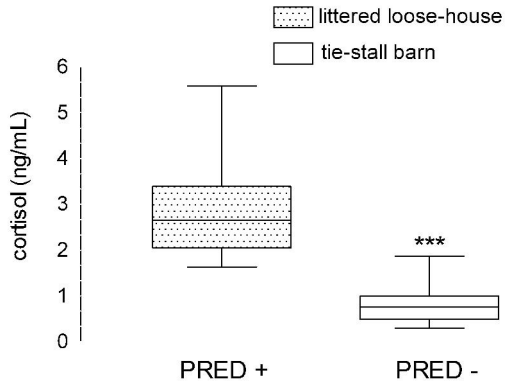

Figure 4. Cortisol levels in cows with (PRED+) or without (PRED-) trace amounts of prednisolone in the same urine samples.